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P01/7700 0.00-0214993.8

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1/77

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1. Your reference 45792 P/63705.CBA/VISD
2. Patent application number  
(The Patent Office will fill in this part) 0214993.8 28 JUN 2002
3. Full name, address and postcode of the or of  
each applicant (underline all surnames)  
Marconi Applied Technologies Limited  
One Bruton Street  
London  
W1X 8AC
- Patents ADP number (if you know it) 7803513001
- If the applicant is a corporate body, give the  
Country/state of its incorporation United Kingdom
4. Title of the invention ELECTROCHEMICAL SENSING
5. Name of your agent (if you have one)  
N. Hucker  
Marconi Intellectual Property  
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6. If you are declaring priority from one or more  
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give the number and the filing date of  
the earlier application
- | Number of earlier application | Date of filing<br>(day / month / year) |
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8. Is a statement of inventorship and of right  
to grant of a patent required in support of  
this request? (Answer 'Yes' if:  
a) any applicant named in part 3 is not an inventor, or  
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11. I/We request the grant of a patent on the basis of this application.

Signature *Nerys Hacker* Date *28/06/02*

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DUPLICATE

## Electrochemical Sensing

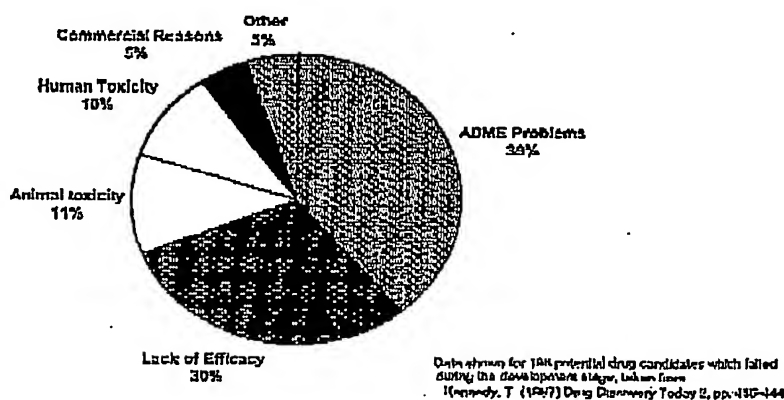
### Summary

A key area of interest in the pharmaceutical industry is the prediction of how drugs are metabolised in the body. One of the main drug metabolism processes, phase I oxidation, is mediated by either the cytochrome P450 [CYP] or flavin monooxygenase (FMO) families of enzymes. The reactions catalysed by these enzymes can be driven electrochemically, and the reaction progress may be monitored using simple electrode systems. This makes the CYPs, and the functionally-related FMOs ideal candidates for electrochemical sensing.

### Background

The single most important decision in the drug design process is made when selecting which of the lead compounds identified during the research programme are to be passed into the development pipeline. About 90% of development candidates fail to become marketed drugs, for a variety of reasons (see figure, below). The cost of a development programme is extremely high (typically £50M per molecule), and so the selection of development candidates has a high financial penalty if made incorrectly. For this reason, there is intense interest within the pharmaceutical industry for effective means to 'fail drugs early', identifying compounds that are unlikely to make it to market before vast expenditure is incurred.

Causes of Attrition in Drug Development



The greatest single reason for a drug candidate to fail during development is for it to show unacceptable characteristics when introduced into live animals or humans. The collective term for these characteristics is ADME/Tox (absorption, distribution, metabolism, excretion and toxicity), covering how well a molecule enters the body, is distributed among the various tissues, is biochemically processed, and then eliminated in the bile or urine, as well as any unexpected toxicological effects that may be uncovered during the development and clinical trials programmes. ADME/Tox prediction is a current area of intense research, and will be an expanding market over the next 3-5 years.

Electrochemical sensing has a role to play in several areas within the ADME/Tox area, since many of the drug metabolism processes involve changes in redox potential. It therefore provides a means to quantify drug molecules, and their metabolic effects, in the context of a whole tissue or body fluid sample. In particular, it provides a sensitive and cost-effective means to follow the metabolic processing of drugs either at the single-enzyme, or whole organ level.

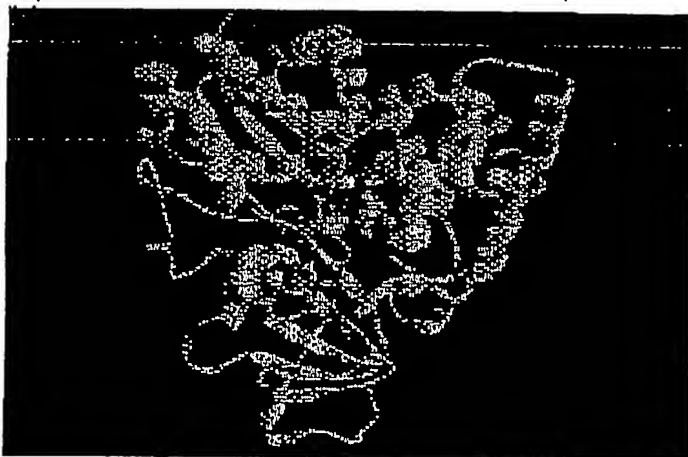
The drug-metabolising enzymes [DMEs] are a diverse group of proteins that are responsible for detoxifying a vast array of xenobiotic compounds ('foreign molecules') including drugs, pesticides and environmental pollutants. Most have an extremely broad substrate specificity: some individual members of the cytochrome P450 [CYP] and flavin monooxygenase [FMO] families are known to metabolise more than 50 structurally diverse compounds. Understanding the structure-activity relationships for the DMEs and their substrates is an important area of research that impacts on pharmacology, toxicology, and basic enzymology. In particular, the ability to predict whether a molecule is likely to be processed by CYPs in the body is of crucial importance in selecting candidate drug molecules for pharmaceutical development.

Conceptually, the DMEs are divided into two groups. Oxidative drug-metabolising enzymes, which include CYPs and FMOs, catalyse the introduction of an oxygen atom into substrate molecules, generally resulting in hydroxylation or demethylation. These enzymes are redox-driven, and the reactions they catalyse may readily be followed electrochemically. The conjugative enzyme families, which include the UDP-glycosyltransferases, glutathione transferases, sulphotransferases, and N-acetyltransferases, catalyse the coupling of endogenous small molecules to xenobiotics. This usually results in the formation of soluble compounds that are more readily excreted. The conjugative enzymes are not redox-driven, and are therefore not particularly suitable for electrochemical sensing. Other, as yet unidentified DMEs may also be found as a result of the human genome project. For example, the recently-discovered CYP3A34 has now been shown to be expressed in certain body tissues, although its precise function is currently unclear. The discussion here is therefore not limited to the enzymes explicitly mentioned in the text.

The CYP and FMO oxidative drug-metabolising enzymes are of particular interest as the biosensor components of an electrochemical device since the electrons they require to drive the reactions can be supplied by direct charge transfer from electrodes in a bioreactor chamber. Indeed, in such a device there is no requirement for additional biological or chemical components such as the cofactors and auxiliary oxidoreductase enzymes that are necessary for driving the reactions in a conventional *in vitro* assay.

## Proposed Biosensor Components

### Cytochrome P450



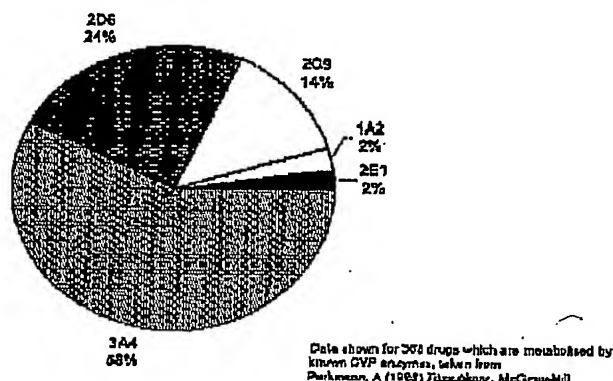
3D molecular structure of CYP2C9 showing the haem group, active-site iron atom and a bound substrate.

CYP enzymes catalyse the initial step in the biotransformation of xenobiotic compounds, including most drugs (a process referred to as first-pass or Phase I metabolism). These enzymes are members of a large family of mixed-function oxidases which typically introduce an oxygen atom into substrate molecules, hence facilitating further metabolic processing to break the compound down. More than fifty CYP isozymes are known to exist in humans and they have been classified into 17

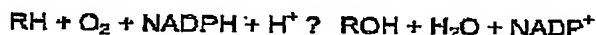
families and 39 subfamilies. In the standard nomenclature, the family is designated by a number, a letter designation for the subfamily, and a second number that identifies the individual member of that subfamily.

In humans and animals, the bulk of drug metabolism is carried out by just a few members of the CYP1, 2, and 3 families and occurs primarily in the liver, which contains the highest concentration of CYP in the body. The figure below shows the percentage of drugs metabolised by the different CYP families. CYP3A4, 2D6, 1A2 and 2C9 are responsible for most of the drug metabolism by CYPs in humans, so are considered the most interesting from a drug screening point of view.

Drug Processing by the Major CYP Isozymes



The oxidation of organic molecules by CYPs is quite complex, but the overall reaction can be simplified to the following equation:



An electron from the cofactor NADPH (a common electron-transfer molecule) is transferred to a haem group within the CYP, where the activation of molecular oxygen occurs. Substrates (represented as R in the above equation) react with one of the oxygen atoms whilst the other is reduced to water, requiring a second electron. Several studies have shown that the electrons necessary to drive this reaction can be supplied electrochemically, with direct charge transfer coming from electrodes in an anaerobic bioreactor. In this case, there is no requirement for the NADPH cofactor. CYP enzymes are therefore ideal candidates for incorporation into an electrochemical sensor for predicting drug metabolism.

## Flavin Monooxygenases

Flavin monooxygenases, like the CYP enzymes, catalyse the oxidation of organic compounds using molecular oxygen and NADPH as the source of electrons for the reduction of one of the oxygen atoms. However, they are mechanistically distinct from the CYPs in that they react with oxygen and NADPH in the absence of substrate to adopt an activated state within the cell, and an interaction with a nucleophilic group such as an amine, thiol, or phosphate is all that is required for completion of the catalytic cycle.

The capacity to remain stable whilst poised in an activated state is a possible explanation for the extremely broad substrate specificity of the FMO isozymes. It has been proposed that essentially all of the energy required for catalysis is captured in the oxygen-activated intermediate, and that alignment or distortion of

the substrate molecules is not required, unlike most other enzymes. It follows that the active sites of FMOs are much less sterically defined than for other enzymes, allowing a wide variety of molecules to act as substrates. FMO3 is the most abundant form in human liver and is believed to be the dominant member of this enzyme family in terms of overall drug metabolism.

As for CYPs, it is possible to drive the FMO-mediated reactions by supplying electrons electrochemically, and therefore these would also be ideal candidates for incorporation into an electrochemical sensor device for predicting drug metabolism.

## Protein-Electrode Interactions

### Immobilised Proteins

One of the most important aspects of driving an enzyme-catalysed reaction electrochemically is the efficient transfer of electrons from the electrode(s) to the catalytic site within the enzyme. One way to maximise this transfer is to immobilise the enzyme at the surface of the electrode. Although a system involving a solubilised enzyme could be designed, early success is most likely by using surface-immobilised enzymes.

### Covalently-modified Electrodes

The surface of an electrode of, for example, metal (typically, though not exclusively gold) or graphite, can be modified by the covalent addition of chemical groups to make it more amenable for the transfer of electrons to proteins. One technique involves the use of organothiolate compounds (containing an SH group) in conjunction with a gold electrode. The thiol group forms a strong bond to the metal surface, with the rest of the molecule providing suitable functional groups for interacting with the protein.

### Microporous Electrolyte Membranes

These are mechanically and chemically stable polymer gels with high ionic conductivity, coating the surface of an electrode in the form of a thin layer. The polymers comprising the gel should be chosen to provide a suitable environment for trapping the proteins within their matrix, such as a high proportion of carboxylic acid groups (for proteins with many positively-charged surface residues), amine groups (for proteins with many negative charges at the surface), or aliphatic groups (for proteins with largely hydrophobic surfaces).

### Lipid Membranes

Natural CYP enzymes are usually found attached to biological membranes, since they almost exclusively contain a region which acts as an anchor within a phospholipid bilayer. Indeed, the CYPs used in analytical laboratories are generally modified to remove this anchor domain, thus allowing the enzyme to be solubilised. The affinity of CYPs for lipid bilayer membranes provide a means of anchoring them at the surface of an electrode. Suitable membranes may be constructed using long-chain fatty acids, lipids, or similar molecules, deposited on the surface.

## Application Areas

Ideally, a drug development team would like to have a detailed picture of the pathway and kinetics of a compound's metabolism in humans, including possible side effects such as CYP induction/inhibition and the generation of toxic metabolites, before beginning clinical trials. Gathering as much of this data as possible usually involves a combination of increasingly targeted assay systems. Whole animals are often used for initial toxicological assessment and the outcome of these experiments can prevent a compound from entering the next phase even before any metabolism work is done. Such studies are currently examined using cultured liver cells, live animals or liver slices in combination with a variety of analytical methods to determine the overall metabolic profile. Most recently, such assays are performed using microsomes, synthetic cells comprising isolated enzymes held in an artificial membrane.

Even with the application of increasingly sophisticated analytical methods, there are obvious difficulties in using animals, cells, or cell fractions to obtain information on the specific biochemical events that comprise a compound's metabolism. Advances in the molecular genetics and biochemistry of the DMEs, and the need for greater efficiency in the drug discovery process are driving the development of new *in vitro* methods based on isolated DMEs. These methods have been used for screening thousands of compounds, and are amenable to integration into the early phases of the drug discovery process. Some of the ways in which recombinant CYPs have been used for *in vitro* metabolism studies and the rationale for these are described in the following sections. The same general approaches can be applied to other DMEs such as the FMOs, but in most cases the methods are not nearly as well developed as they are for the CYPs.

## Isozyme Identification

An identification of the major enzyme(s) involved in your specific drug's metabolism is perhaps the most important component of early studies. Once this is known, pharmacokinetic [PK] studies (see below) are done to obtain  $K_m$  (an approximate measure of the affinity of the enzyme for the substrate) and  $V_{max}$  (how fast the enzyme can process substrate molecules). Together, these parameters are used to estimate *in vivo* clearance rates, a key determinant of therapeutic efficacy. Knowledge of the metabolism rate by a specific enzyme may alert the drug discovery team to potential pharmacogenetic problems or drug-drug interactions.

Genetic differences in CYP levels are a major cause of individual variability in response to therapeutics. For example, roughly 8% of the Caucasian population are poor metabolisers of 2D6 substrates and can experience serious side effects when administered normal doses of drugs that are metabolised primarily by this isozyme. Furthermore, some drug-drug interactions can cause serious side effects or even fatal conditions such as drug-induced arrhythmia. The identification of which enzyme is primarily responsible for the metabolism of a drug aids in the design of effective clinical studies used for assessing possible drug interactions. A panel of CYP and FMO enzymes used as biosensors would enable the degree of processing of a new drug by each isozyme to be accurately quantified.

## Determination of Kinetic Parameters

Undesirable PK characteristics are frequently a factor in the failure of compounds in preclinical studies. The goal of *in vitro* studies is to determine the key PK parameters ( $K_m$  and  $V_{max}$ ) for a compound with each CYP isozyme in order to obtain an estimate of the overall *in vivo* clearance rate. The problems with attempting to obtain accurate kinetic data from crude enzyme preparations such as microsomes include metabolism of the substrate by more than one isozyme, further modification of products (e.g. conjugation), consumption of NADPH by contaminating redox enzymes, and binding of substrates or products to cell



proteins or other macromolecules. From an enzymologist's point-of-view, the only way to obtain accurate kinetic data is with isolated enzyme systems. Isolated CYP enzymes used as biosensors would provide this capability.

## High-Throughput Screening

A large number of pharmacologically active compounds synthesized in the discovery phase of pharmaceutical R&D are rejected because they interact with the metabolism of existing therapeutic drugs or because they have poor bioavailability caused by rapid metabolism. In many cases, this is because the compounds are either substrates or inhibitors of one or more CYP isozymes. CYPs and other DMEs are generally assayed by isolation and quantification of the metabolites produced from the parent compound. In most cases, this involves chromatographic techniques (usually HPLC) and in some cases phase separations. There are two major drawbacks to these assay methods. First, the need to isolate the reaction products makes the methods too cumbersome and time consuming for use in any type of high-volume assay and precludes the collection of continuous kinetic data. Second, measurement of metabolites requires use of different assay methods for every substrate, raising an obvious technical barrier to screening diverse compounds for metabolism. A universal assay method would be ideal in that it would allow direct quantification of metabolism rates for any substrate, allowing the determination of the key pharmacokinetic parameter (calculated as  $V_{max}/K_m$ ) for diverse compounds in a high-throughput screening [HTS] format. The intuitive approach for achieving this is to monitor NADPH consumption, which theoretically should be stoichiometric with substrate turnover. However, this has not proven practical because the coupling between NADPH consumption and substrate turnover is variable between different substrates and is frequently as low as 20-30%. Measurement of oxygen consumption suffers from the same drawback; a significant percentage of the total oxygen consumed is diverted into reactive oxygen intermediates rather than metabolite and water.

For these reasons, the main approach that is currently used for screening is competitive inhibition assays, in which inhibition of a probe substrate turnover by the test compound is used to identify potential substrates and inhibitors. The hits from these competitive inhibition screens must be further evaluated to determine whether they are inhibitors or substrates for the indicated isozyme. A number of approaches have been developed for high-throughput screening of CYP inhibition. These techniques include rapid phase separation methods for isolating radiolabeled CYP 2D6 metabolites, development of robotically controlled, multi-column HPLC separation systems to assay testosterone metabolism by CYP 3A4, the use of novel chromogenic reagents for quantitation of formaldehyde formation during CYP-dependent demethylation reactions, and rapid LC/MS approaches for metabolite analysis. However, all of these approaches include relatively cumbersome post-reaction separation steps that limit their usefulness in an HTS format. A lab-on-a-chip style biodetector able to follow CYP mediated reactions at the pharmacokinetic level would not require these separation steps, and so would offer substantial benefits over the current HTS technologies.